Exhibit D

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THE CELL BIOLOGY OF INFECTION BY INTRACELLULAR BACTERIAL PATHOGENS

Julie A. Theriot
Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142

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ABSTRACT

Listeria monocytogenes and *Shigella flexneri* are unrelated bacterial pathogens that have independently evolved similar strategies of survival within an in-

fected host animal. Bacteria coming into contact with the surface of an epithelial cell induce cytoskeletal rearrangements resulting in phagocytosis. They then secrete enzymes that degrade the phagosomal membrane, releasing the bacteria into the host cell cytoplasm. Intracytoplasmic bacteria move rapidly, in association with a "comet tail" made up of host cell actin filaments. When moving bacteria reach the cell margin, they push out long protrusions with the bacteria at the tips that are then taken up by neighboring cells, allowing the infection to spread from cell to cell. This review summarizes what is currently known about the interactions between the bacteria and the host at each stage of the infection and discusses what mammalian cell biologists can learn by studying bacterial pathogens.

INTRODUCTION

One difficulty in mammalian cell biology is the frequent lack of appropriate agents for manipulating basic cell processes. Traditionally, cell biologists have relied heavily on pharmacology, but drugs are rarely perfectly specific in their actions, and for many interesting cell behaviors, no useful drugs are available. Few mammalian systems are amenable to classical genetic analysis. Recently developed reverse genetic techniques have been used productively, but these generally require prior identification and molecular cloning of a protein of interest, and often some prior understanding of its function. One relatively underexplored area that can provide more tools for investigators is the interaction between pathogenic microorganisms and their mammalian hosts. The study of viral reproduction has helped us more completely to understand mammalian host cell processes ranging from the replication of DNA to the sorting of plasma membrane proteins. Similarly, studies of bacterial and eukaryotic parasites can yield a wealth of information about the host. The idea that cell biologists can make use of pathogenic organisms to study basic cellular processes has recently spread. Recently, a volume of the series *Methods in Cell Biology* was entirely devoted to the use of microbial pathogens (Russell 1994). In this article, I focus on bacterial pathogens and how they can help us to understand the workings of the mammalian cell cytoskeleton.

The manifestation of disease is rarely due simply to the presence of bacteria infecting a human. Rather, symptoms are caused by the response of host cells to specific signals generated by the bacteria. In order to cause disease, then, a pathogenic bacterium must be an excellent cell biologist, able to communicate with host cells and manipulate their behaviors. Pathogenic bacteria have coevolved with their hosts and fine-tuned their interactions, and human cell biologists can learn much about basic mammalian cell activities by watching how bacteria have learned to interfere with them.

What Can Cell Biologists Learn from Studying Bacterial Pathogens?

One well-known example of a crucial advance cell biologists owe to pathogenic bacteria is the elucidation of the functions of heterotrimeric G proteins associated with hormone receptors. Cholera toxin, produced by *Vibrio cholerae*, ADP-ribosylates $G_{s\alpha}$, the G protein subunit that activates adenylyl cyclase in response to hormonal signaling through β-adrenergic receptors. ADP-ribosylated $G_{s\alpha}$ is unable to hydrolyze its bound GTP, and therefore constitutively activates adenylyl cyclase, resulting in an overproduction of cyclic AMP and eventally leading to the ion imbalance and water loss of choleral diarrhea (Lai 1980). Although the binding of epinephrine to β-adrenergic receptors normally activates adenylyl cyclase, the same hormone binding to α2-adrenergic receptors causes adenylyl cyclase inhibition. α2-Adrenergic-mediated inhibition is unaffected by cholera toxin, although it is abolished by pertussis toxin, produced by *Bordetella pertussis*, the causative agent of whooping cough. The reason for this difference was made clear by the discovery of a distinct G protein subunit, $G_{i\alpha}$, that associates with α2-adrenergic receptors and inhibits adenylyl cyclase, and can be ADP-ribosylated and inactivated by pertussis toxin. The activity of $G_{i\alpha}$ had been inferred from a variety of indirect experiments, but the identification of the pertussis toxin substrate was the first direct evidence for its existence (Katada & Ui 1982a,b). The characteristic effects of cholera toxin and pertussis toxin are considered important criteria for the involvement of G proteins in a signaling pathway (Gilman 1987), and these two toxins continue to be standard reagents in laboratories studying cell signaling.

The use of purified secreted toxins is only one possible contribution of pathogenic bacteria to cell biology. It is often possible to use the living bacteria themselves as probes for more complex processes. Contact between a pathogenic bacterium and a host cell may lead to various dramatic host-cell responses from rapid cytoskeletal rearrangements, to long-term transcriptional changes, and even programmed cell death. Presentation of the bacteria is a method to manipulate these host cell behaviors in a highly reproducible way. Many bacteria are amenable to genetic analysis, and bacterial factors required for particular specific effects on the host cell usually can be identified. These can provide a molecular handle for identifying host cell proteins directly involved in the regulated behavior.

Types of Interactions Between Pathogenic Bacteria and Host Cells

Bacterial colonization of a host does not automatically lead to disease; the bacteria must elicit specific responses from some particular host cells. Many

bacteria exert their primary effect on the cells of the immune system, for example, by causing inflammation or macrophage activation. In this review, I rely mostly on examples of interactions between bacteria and intestinal epithelial cells in order to emphasize how their interactions are relevant to general cell biology. A useful overview of the pathogenesis of all these examples can be found in Salyers & Whitt (1994). Several different paradigms of interaction exist that can be characterized as advancing levels of intimacy in the host-parasite contact.

At one extreme, it is possible for pathogenic bacteria to exert a specific effect on intestinal epithelial cells without colonizing the host or coming into direct contact with host cells. Two examples are food poisoning caused by *Clostridium botulinum* and *Staphylococcus aureus*. In both cases, the bacteria grow in contaminated food and secrete exotoxins. When the food is consumed by a susceptible host, the toxins cause enteric symptoms with or without the presence of the bacteria.

The next level of contact is when bacteria colonize the surface of host cells and grow there without causing contact-mediated host-cell responses. For example, *Vibrio cholerae* can adhere to the mucosal surface of the small intestine and multiply, although the presence of the bacteria does not directly induce any alteration in the morphology of the host cells. In this case, the specific effects on the signaling apparatus of the host cells are still primarily caused by secreted soluble toxins despite the proximity of the bacteria themselves.

Some pathogens do cause contact-mediated morphological changes in the host cell. Enteropathogenic *Escherichia coli* adhere closely to the epithelial cell apical surface and cause effacement of the microvilli. Dense accumulations of actin filaments and actin-associated proteins are found underneath closely adhering bacteria, forming a pedestal structure that seems to embrace the bacteria without engulfing them. Attachment of these bacteria to the cell surface causes destruction of one type of cytoskeletal structure (microvilli) and creation of a distinct type (pedestals). These complex and specific rearrangements are directed by the bacteria while they remain external to the host cell and communicate through the host cell plasma membrane; direct contact between the bacterial surface and the host cell surface is required.

In contrast to these examples of bacteria that interact with host cells while remaining outside of them, a variety of pathogens actually live inside the cells of the infected host. To enter the host cells, the bacteria must elicit phagocytosis by cells that are not normally phagocytic. This also requires that the bacteria induce rearrangements of the host cell cytoskeleton and remodeling of the cell surface by transmembrane signaling. One widely studied example of this class of bacteria is *Salmonella typhimurium*, which survives and multiplies within a membrane-bound compartment after it is

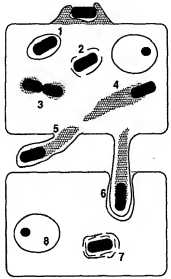
phagocytosed by the cell. This is a common strategy of pathogenic bacteria, also shared by *Mycobacterium tuberculosis* (the causative agent of tuberculosis) and *Yersinia pestis* (the causative agent of plague), among many others. All these pathogens have developed strategies either to prevent the fusion of their initial compartment with lysosomes or to survive in the hostile environment of the phagolysosome.

At the extreme of closest host-parasite contact, a few types of intracellular bacterial pathogens gain access to and grow directly in the cytoplasm of the host cell, rather than remaining within a membrane-bound compartment. The two most often studied members of this class are *Listeria monocytogenes* and *Shigella flexneri*. The remainder of this review focuses on what is known about the pathogenesis of these two examples, with particular emphasis on the interactions between the bacteria and the host cell actin cytoskeleton.

OVERVIEW OF HOST CELL/PATHOGEN INTERACTION CYCLE FOR *L. MONOCYTOGENES* AND *S. FLEXNERI*

S. flexneri is a gram-negative bacterium closely related to *E. coli*. *L. monocytogenes* is gram positive, and has been separated from *S. flexneri* for millions of years of evolution. *S. flexneri* is a common cause of bacillary dysentery in areas lacking a clean water supply. *L. monocytogenes* can cause serious infections including meningitis in immunocompromised people, newborns, and pregnant women, and is usually contracted after eating heavily contaminated food. The two diseases have no obvious similarity beyond the oral route of transmission. However, the interactions of these bacteria with the cells of the infected host share remarkable similarities.

Both bacteria are intracellular pathogens and members of the subset that grow primarily in the cytoplasmic compartment of infected host cells. The pathway by which these organisms gain access to the host cell interior and spread between cells is diagrammed in Figure 1. Bacteria coming into contact with the surface of an epithelial cell induce cytoskeletal rearrangements resulting in phagocytosis. They then secrete enzymes that degrade the endocytic membrane, releasing the bacteria free in the host cell cytoplasm. Once in the cytoplasm, the bacteria begin to grow and divide and are soon coated with a cloud of host cell actin filaments. After some time, this symmetric cloud is rearranged into a polar comet tail made up of short, cross-linked actin filaments (Figure 2). Bacteria associated with comet tails move rapidly throughout the host cell (Figure 3). When moving bacteria reach the cell margin, they keep moving, pushing out long finger-like protrusions with the bacteria at the tips. These protrusions are apparently recognized and phagocytosed by neighboring cells, allowing the infection to spread from cell to cell. Because the bacteria



	L. monocytogenes	*S. flexneri*
1. Attachment and internalization	*inlA*	*ipaBCD*
2. Escape from endosome	*hly, plcA*	*ipaB*
3. Actin filament nucleation	*actA*	*icsA*
4. Tail assembly, movement	*actA*	*icsA*
5. Protrusion formation	?	?
6. Protrusion uptake	?	?
7. Escape from double membrane	*plcB*	*icsB, vacJ*
8. Apoptosis	not applicable	*ipaB*

Figure 1 Interactions between *L. monocytogenes* or *S. flexneri* and a host cell. Actin filament accumulations are marked by shaded areas. Bacterial genes known to be involved in each step are summarized at the right.

are never outside of the cytoplasm, they are not attacked by the host humoral immune system.

Genetic investigations by a number of laboratories have identified bacterial genes involved in most stages of this process (Figure 1). The organization of virulence genes in the two organisms is quite different; *L. monocytogenes* carries all its known virulence determinants on the bacterial chromosome (Michel & Cossart 1992), whereas the majority of *S. flexneri* virulence genes are found on the large plasmid associated with all pathogenic *Shigella* strains and enteroinvasive *E. coli* (Kopecko et al 1985, Yoshikawa & Sasakawa 1991). There is generally no identifiable sequence similarity between *L. monocytogenes* virulence genes and their *S. flexneri* equivalents. The two organisms seem to have independently evolved their similar styles of interaction with host cells. The genetic basis of pathogenesis has been recently reviewed for *L. monocytogenes* (Portnoy et al 1992a, Sheehan et al 1994) and *S. flexneri* (Yoshikawa & Sasakawa 1991, Hale 1991, Sansonetti 1991). Therefore, in this review I focus on the points of interaction between the infecting bacteria and their eukaryotic hosts and what is known about the cell biology of each step.

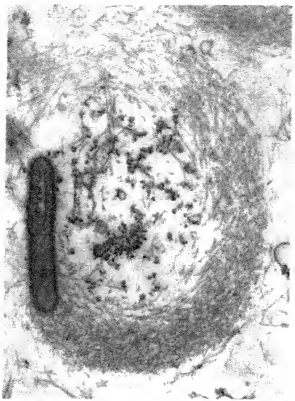


Figure 2 Thin-section electron micrograph from an infected tissue culture cell showing *L. monocytogenes* associated with an actin-rich comet tail. Micrograph courtesy of M Siebert.

ENTRY INTO HOST CELL AND ESCAPE INTO CYTOPLASM

S. flexneri and *L. monocytogenes* have developed strategies of survival within an infected animal that require their access to the cytoplasm of the host cells. Initial infection of the gut epithelium requires entry into the colonic epithelial cells. Entry can be summarized as three sequential steps: first attachment of the bacteria to the host cell surface, then internalization of the bacteria by the host cell, and finally degradation of the endocytic membrane, which allows the bacteria to enter the cytoplasm.

Attachment and Internalization

These two organisms share with many other intracellular pathogens the ability to induce uptake by cells that are not normally phagocytic (reviewed in Falkow

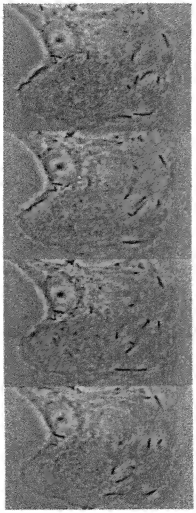


Figure 3 Movement of *L. monocytogenes* inside an infected tissue culture cell. Four frames are shown from a video sequence, separated by 30-s intervals. Individual bacteria are black oblongs, comet tails are visible as phase-dense streaks behind the moving bacteria.

1991, Falkow et al 1992, Rosenshine & Finlay 1993). Parasite-directed phagocytosis generally requires that the bacteria first adhere to the surface of the host cell and then induce cytoskeletal rearrangements resulting in engulfment.

L. monocytogenes appear to enter intestinal epithelial cells through the apical side. Prior to internalization, the attachment of the bacteria to the cell surface causes modification of the structure of the microvilli (Karunasagar et al 1994). The protein internalin, the product of the *inlA* gene, is required for both attachment and uptake. Mutant strains in which the gene has been disrupted

cannot invade cells: Expression of the protein in the nonpathogenic species *L. innocua* confers on it the ability to (inefficiently) invade tissue culture cells (Gaillard et al 1991). It is probable that other *L. monocytogenes* gene products contribute to this process. As is true of most phagocytic events, actin polymerization is required for bacterial entry (Gaillard et al 1987). The host cell receptors that recognize internalin have not been identified, and little is known about the downstream signaling events that result in phagocytosis. It has recently been reported that mitogen-activated protein (MAP) kinase is stimulated in the host cell when *L. monocytogenes* adhere to the surface and that inhibitors of tyrosine kinases block bacterial uptake (Tang et al 1994). How *L. monocytogenes* attachment activates MAP kinase and how the kinase contributes to cytoskeletal rearrangements and bacterial entry have yet to be determined.

S. flexneri cannot efficiently enter cells through the apical pole but do enter readily through the basolateral pole (Mounier et al 1992). In an infected animal, the bacteria must find a way through the tight junctions between cells of the intestinal epithelium to access their basolateral regions. Recent evidence suggests that local inflammation and polymorphonuclear leukocyte transmigration through the gut epithelium, induced by the presence of *S. flexneri* on the apical surface, can sufficiently disrupt the tight junctions to allow bacteria to pass through (Perdomo et al 1994a,b). Actin polymerization is required for *S. flexneri* invasion into cells (Hale et al 1979), and myosin is accumulated at the site of bacterial attachment (Clerc & Sansonetti 1987).

The genetic basis of invasion is substantially more complex for *S. flexneri* than for *L. monocytogenes*. Multiple genes in the *ipa* operon are required for *S. flexneri* entry into epithelial cells. The highly antigenic secreted Ipa protein products lack a standard signal sequence and require a complex, specialized secretory apparatus to exit the bacterium. Many mutations in genes of the *mxi* and *spa* operons, which encode this virulence-factor secretion apparatus, cause *S. flexneri* to become noninvasive because of their effects on Ipa protein secretion (Andrews et al 1991, Allaoui et al 1992b, 1993; Venkatesan et al 1992). The virulence-specific secretory pathway of *S. flexneri* has homologues in both *Yersinia* and *Salmonella* species, but the pathway is absent in *E. coli* (Maurelli 1994). This has made it difficult to express Ipa proteins in a functional form in *E. coli*, and the functional analysis of these proteins has been limited. Some information about the roles of the four Ipa proteins has been obtained with the use of nonpolar mutants. IpaA is a major antigen in infected hosts, but strains failing to express it appear to be fully invasive, and its function is unknown. In contrast, strains lacking any of IpaB, IpaC, and IpaD are unable to enter tissue culture cells, although they can adhere to the cell surface (Ménard et al 1993). Thus IpaB, IpaC, and IpaD must be directly or indirectly required for parasite-mediated phagocytosis, but the initial attachment of bac-

teria to the host cell requires other unidentified factors. IpaB and IpaC form a heterocomplex after secretion. They are prevented from prematurely associating with one another in the bacterial cytoplasm by the binding of IpgC, a distinct protein that appears to act as a molecular chaperone for IpaB and IpaC. Bacteria lacking IpgC cannot invade epithelial cells, presumably because IpaB and IpaC are not properly secreted in its absence (Ménard et al 1994). The host cell receptors for IpaB, IpaC, and/or IpaD have not been identified, and the mechanism by which the *S. flexneri* proteins induce the cytoskeletal rearrangements that result in phagocytosis is unknown.

Escape from Membrane-Bound Compartment

Once the infecting bacteria have been internalized by a host cell, they must escape from the phagosome to gain access to the cytoplasmic environment. As mentioned above, numerous pathogenic bacteria have developed strategies for survival and growth in the phagosome or phagolysosome, but *L. monocytogenes* and *S. flexneri* are not able to multiply within the membrane-bound compartment (Gaillard et al 1987, High et al 1992). Both organisms, therefore, produce enzymes that partially degrade the phagocytic membrane, allowing the bacteria to enter the cytoplasm.

Listeriolysin O was the first virulence factor identified for *L. monocytogenes* (Gaillard et al 1986). It is very similar to other members of its family of pore-forming hemolysins of which the best known is streptolysin O (Smyth & Duncan 1978). Strains that do not produce listeriolysin O are taken up by mammalian cells but generally remain within the phagosome (Gaillard et al 1987, Kuhn et al 1988) and are essentially avirulent in animal models (Gaillard et al 1986, Kathariou et al 1987, Portnoy et al 1988). Transfer of the gene encoding listeriolysin O, *hly*, into *Bacillus subtilis* confers both hemolytic activity and the ability to enter and grow within the cytoplasm of macrophage-like tissue culture cells (Bielicki et al 1990).

It is surprising that intracellular bacteria can express high levels of a pore-forming hemolysin without killing their host cells by perforating the plasma membrane. The enzymatic activity of listeriolysin O is highest at acidic pH such as is found in endosomal compartments but lower at neutral (cytoplasmic) pH (Geoffroy et al 1987); this low pH optimum is not shared by other members of the hemolysin family, which are generally active over a broader pH range. In an infected cell, it would be expected that the activity of listeriolysin O is high when the bacteria are first in the acidifying phagocytic vacuole and is lower when the bacteria have escaped into the cytoplasm, so the secreted enzyme ruptures the endosomal membrane without rupturing the plasma membrane. Consistent with this idea, *B. subtilis* or *L. monocytogenes* expressing perfringolysin O in place of listeriolysin O are able to escape the endosome and replicate briefly in the cytoplasm, but the infected cells soon die (Portnoy

et al 1992b, Jones & Portnoy 1994). The evolution of the pH-sensitive listeriolysin O from its pH-insensitive relatives is one example of how *L. monocytogenes* has "learned" enough about mammalian cell biology to enhance its survival in the host.

In addition to listeriolysin O, *L. monocytogenes* also secretes two phospholipases, a phosphatidylinositol-specific phospholipase C encoded by *plcA* (Leimeister-Wächter et al 1991, Mengaud et al 1991a) and a broad-range phospholipase C encoded by *plcB* (Vazquez-Boland et al 1992). *plcA* was initially thought to be a critical virulence gene because strains carrying transposon insertions in that gene are essentially avirulent and incapable of invading tissue culture cells (Camilli et al 1991, Mengaud et al 1991a). The avirulence of these strains, however, was not due to the disruption of *plcA*, but rather to a polar effect on a downstream gene *prfA*, which encodes a transcriptional activator necessary for the positive regulation of all other known virulence genes including *hly* (Leimeister-Wächter et al 1990, Mengaud et al 1991b). Subsequent experiments using in-frame internal deletions have shown that the phosphatidylinositol-specific phospholipase C is not generally required for entry into cells but that it may assist in rapid exit from the phagocytic vacuole in primary mouse macrophages (Camilli et al 1993). Expression of the *L. monocytogenes* phosphatidylinositol-specific phospholipase C in *L. innocua* does not confer the ability to escape into the cytoplasm, but it does allow the bacteria to replicate for a few generations within the phagolysosome, suggesting that it may partially disrupt the phagocytic membrane enough to prevent immediate bacterial killing but not enough to allow bacterial exit (Schwan et al 1994).

S. flexneri also possess a contact-mediated hemolytic activity (Sansonetti et al 1986) that is enhanced at acidic pH (Clerc et al 1986). Strains with disruptions in the *ipaB* gene (described above as being important in directing parasite-mediated phagocytosis) are unable to escape from the phagosomes of macrophage-like cells and lack contact hemolytic activity (High et al 1992). IpaB is not similar in sequence to the streptolysin O family of hemolysins, nor to any other known hemolysins (Baudry et al 1988), and its enzymatic activity is unknown. It is not yet clear whether other *S. flexneri* gene products act with IpaB to enable disruption of the phagocytic membrane and escape of the bacteria into the host cell cytoplasm.

Neither *S. flexneri* nor *L. monocytogenes* appears to require specialized genes for survival in the cytoplasm once they have escaped from their initial membrane-bound compartments. Indeed, the mammalian cytoplasm appears to be a good medium for bacterial growth. The doubling rate of the bacteria in infected cells is similar to the rate in rich broth. *B. subtilis* and *E. coli* obviously do not normally encounter cytoplasm, but if they are given access to it (by expressing the necessary proteins from *L. monocytogenes* or *S. flexneri*,

respectively) they are capable of multiplying there (Bielicki et al 1990, Sansonetti et al 1983). Furthermore, many auxotrophic *L. monocytogenes* mutants grow in cytoplasm at normal rates, and are virulent (Marquis et al 1993). It is not clear why so few pathogenic bacteria have developed infection strategies that include growth directly in host cell cytoplasm.

INTERACTIONS WITH THE ACTIN CYTOSKELETON AND MOVEMENT OF BACTERIA THROUGH THE CYTOPLASM

Once the bacteria are in the host cell cytoplasm, they require some form of motility to efficiently spread within and between infected cells. Bacterial flagella would not be useful for movement through the extremely viscous cytoplasm. As with any cellular organelle, intracellular bacteria are expected to require association with the cytoskeleton in order to move. In theory, the bacteria might move around within a single cell by tracking along preexisting cytoskeletal structures such as microtubules or actin stress fibers. Intracellular *S. flexneri* have been observed to move parallel to actin stress fibers in cultured avian fibroblasts (Vasselon et al 1991), but it is not clear how important this type of movement is in vivo. The majority of intracytoplasmic movement performed by *L. monocytogenes* and *S. flexneri* is due to the bacterially induced formation of phase-dense, actin-rich comet tails (Figures 2, 3) (Bernardini et al 1989, Pal et al 1989, Tilney & Portnoy 1989, Mounier et al 1990, Dabiri et al 1990). Rapid movement of the bacteria at the front of the comet tails allows efficient spread throughout an infected cell and also results in the formation of protrusions that are necessary for cell-to-cell spread.

Structure and Components of the Actin-Rich Comet Tail

The comet tail structures have been intensively studied by several laboratories over the past few years, largely prompted by the thorough electron microscopic study of intracellular *L. monocytogenes* by Tilney & Portnoy (1989). This study demonstrated that actin filaments initially coat the bacteria in symmetric clouds, but as the bacteria grow in the cytoplasm over the first few hours of infection, the clouds rearrange to form asymmetric tails. Bacterial division seems to be involved in the evolution of tail asymmetry. Even early in infection, there are more actin filaments associated with the poles of a dividing bacterium than with its middle. Thus as the two daughter cells break apart, one pole of each (the old pole) is associated with a nascent comet tail, while the other pole (the new pole) is denuded of actin filaments (Tilney & Portnoy 1989). Similarly, when the movement of newly divided bacteria is observed using videomicroscopy, the two daughter cells invariably move in opposite directions, with the newly formed pole leading (JA Theriot, unpublished observations).

After a few rounds of bacterial division in the infected host cell, most intracytoplasmic bacteria are associated with the asymmetric comet tails (Tilney & Portnoy 1989), and typically up to 80% of the bacteria in a cell are moving (Theriot et al 1994). The filaments appear to be only loosely oriented in the tail meshwork, although there is some preference for the filaments to align parallel to the long axis of the tail at the bacterial surface and along the shell of the tail (Zhukarev et al 1995). Actin filaments in the comet tails are generally short, with an average length of approximately 0.3 μm (about 100 monomers) (Tilney & Portnoy 1989) (Figure 3). When these filaments are decorated with the S1 head fragment of skeletal muscle myosin, the arrowheads formed by S1 most often point away from the bacteria; this indicates that the rapidly growing barbed end of the polar actin filament is associated with the bacterial surface (Tilney et al 1992). In most mammalian nonmuscle cells, approximately half the actin is polymerized in filamentous form and half is in monomeric form at any given time (Korn 1978, Heacock & Bamburgh 1983). The pool of sequestered actin monomers is prevented from polymerizing by monomer-binding proteins including profilin and thymosin β_4 (reviewed in Fechheimer & Zigmond 1993). Regulated desequestration of actin monomers can allow filament growth during rapid cytoskeletal rearrangements (reviewed in Theriot 1994). In theory, the actin filaments of the *L. monocytogenes* comet tail could either be recruited from other filamentous structures in the cell or assembled by de novo actin polymerization in the tail. Filaments stabilized by the fungal toxin phalloidin cannot be recruited into newly forming tails (Sanger et al 1995), thus the tails are apparently generated by actin polymerization from the monomer pool.

A variety of actin-associated proteins are found in the comet tails associated with *L. monocytogenes* and *S. flexneri*. These proteins include tropomyosin (Dabiri et al 1990), the actin filament bundling proteins α-actinin (Dabiri et al 1990) and fimbrin (Prévost et al 1992, Kocks & Cossart 1993), and the adhesion plaque proteins vinculin and talin (Kadurugamuwa et al 1991, Dold et al 1994). There are probably many other actin-associated proteins enriched in the tails, possibly including factors that bind to and cap the barbed or pointed ends of the filaments (Tilney et al 1992). It is difficult to determine which of these actin filament-binding proteins are required for tail formation and motility, and which are present simply because of their affinity for actin. It seems reasonable to assume that at least some of the cross-linking proteins are necessary to create and maintain the structure of the tail. In the case of *L. monocytogenes*, it has recently been demonstrated that microinjection of proteolytic fragments of α-actinin into infected cells causes dissolution of tails and cessation of bacterial movement (Dold et al 1994). Actin filament clouds are still associated with the bacteria, but the microinjected α-actinin fragments block filament cross-linking, and without this component of tail formation,

movement cannot be generated. The actin monomer-binding protein profilin and the actin filament-associated vasodilator-stimulated phosphoprotein (VASP) are the only known host proteins localized at the bacterial surface that are not present throughout the rest of the tail (Theriot et al 1994, Chakraborty et al 1995); profilin and VASP may be involved in actin filament nucleation or growth (see below).

Actin Filament Dynamics in the Comet Tail

It was not clear from the static electron microscope pictures alone whether the comet tails are stable structures that move through the cytoplasm with the bacteria, or whether the filaments in the tail remain stationary in the cytoplasm, growing at the front of the tail and depolymerizing elsewhere. Subsequent dynamic experiments have supported the second hypothesis. Filaments in the tails of moving bacteria, marked by fluorescence photoactivation, remain stationary in the cytoplasm as the bacteria move away (Theriot et al 1992). Furthermore, exogenous actin monomers are preferentially incorporated into the tails near the bacterial surface, both in living and in permeabilized cells (Sanger et al 1992, Tilney et al 1992). Quantitative analysis of these experiments indicates that the rate of actin filament growth at the bacterial surface can entirely account for the movement rates observed and that actin polymerization does not occur anywhere else in the comet tail.

Actin filaments in the tail have an average half-life of 30 s to 1 min (Theriot et al 1992, 1994; Sanger et al 1992, Nanavati et al 1994). The rate of actin filament loss is independent of position in the tail and of bacterial speed (Theriot et al 1992); this stochastic filament disappearance results in an exponential decay of filament density in the tail both in time and in space (Theriot et al 1992, 1994). As a bacterium stops moving, the filaments in the tail continue to disappear at the same rate, resulting in an apparent shortening of the tail. Generally, filament turnover is regulated by the steady-state behavior of the actin cytoskeleton of the host cell and is not significantly affected by the rate of bacterially directed filament growth at the front end. Thus rapidly moving bacteria tend to be associated with longer tails because they move further away from a newly created segment of the tail before it disappears (Theriot et al 1992, Sanger et al 1992). *L. monocytogenes* actin-based motility can be reconstituted in cytoplasmic extracts made from the eggs of the clawed frog *Xenopus laevis*. Comet tails formed in cell-free cytoplasmic extracts behave essentially identically to those in intact cells (Theriot et al 1994). The simplest model of actin filament dynamics in the comet tail of *L. monocytogenes*, consistent with all the observations, is diagrammed in Figure 4.

The dynamic behavior of actin filaments in comet tails associated with *Shigella flexneri* has not been as thoroughly studied. Preliminary results suggest that *S. flexneri* comet tails are very similar to those of *L. monocytogenes*

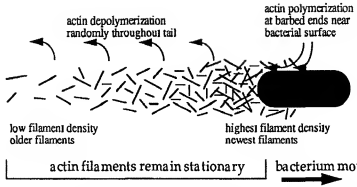


Figure 4 Diagram of actin filament dynamics in *L. monocytogenes* comet tails. Short actin filaments are recruited to or nucleated at the bacterial surface and are rapidly elongated at their barbed ends. The growing filaments are incorporated into a tail structure that is stationary in the cytoplasm, where they become coated and cross-linked by actin-binding proteins. The rate of bacterium movement is equal to the rate of actin filament growth at the front end of the tail. The filaments depolymerize randomly and rapidly.

with respect to filament density distribution and rate of turnover (Goldberg & Theriot 1995). Furthermore, *L. monocytogenes* and *S. flexneri* infecting the same line of tissue culture cells move over an identical range of speeds (JA Theriot, unpublished observations). These observations are consistent with the hypothesis that actin filament polymerization occurs only adjacent to the bacterial surface in the case of *S. flexneri* movement as well as for *L. monocytogenes*. It is reasonable to conclude that steady-state host cytoskeletal dynamics rather than specific bacterially directed regulation control the tail architecture and behavior and that this behavior is essentially identical between *S. flexneri* and *L. monocytogenes*. Therefore, the role of the bacteria is to trigger a process that is inherent to the steady-state regulation of actin filament dynamics in the infected host cells, but this process may be triggered in slightly different ways by the two different organisms (see below).

Bacterial Products Involved in Motility

Because actin filament polymerization occurs only immediately adjacent to the bacterial surface, it is reasonable to assume that factors secreted by the bacterium or expressed on the bacterial surface direct polymerization. Both *S. flexneri* and *L. monocytogenes* can continue to move in infected tissue culture cells for several hours in the presence of drugs that inhibit bacterial protein synthesis (Tilney et al 1992, Goldberg et al 1994). Therefore, it is likely that the host cytoskeleton associates with a stable surface-bound bacterial protein rather than a continually secreted factor. In principle, the bacterial protein may

act by nucleating actin filaments, by locally uncapping barbed ends of preex isting filaments, by promoting rapid filament elongation (perhaps by locally desequestering actin monomer), or by providing a binding site for a hos cytoskeletal protein that performs one of these functions.

Transposon mutagenesis has been used in both organisms to identify viru lence factors that are important for their associations with the actin cytoskele ton. In *L. monocytogenes*, mutant strains in which the *actA* gene has beei disrupted by insertions (Kocks et al 1992, Domann et al 1992) or in-fram internal deletion (Brundage et al 1993) can invade cells and grow normally but are unable to associate with actin and are significantly deficient in cell-to cell spread and virulence. *actA* encodes a transmembrane protein with a stand ard signal sequence at the amino terminus and a membrane anchor domai near the carboxy terminus (Kocks et al 1992, Domann et al 1992). Insid infected cells, the protein is completely associated with the bacterial surface it is not found throughout the comet tails (Niebuhr et al 1993, Kocks et a 1993). The ActA protein becomes phosphorylated at several sites by kinase in the infected host cell (Brundage et al 1993). The function of this phospho rylation is unknown.

Along with the phenotype of *actA*-deficient mutant strains, several othe lines of evidence suggest that ActA is directly involved in the association o *L. monocytogenes* with the host cell actin cytoskeleton. ActA is distributed i a gradient on the surface of *L. monocytogenes*, growing in broth or in infectec host cells. In infected cells, the end of the bacterium with the higher concen tration of ActA is invariably the end associated with the comet tail. On dividin bacteria, ActA is specifically depleted from the septation zone, so the protei is most highly concentrated at the old pole of a newly separated daughter cel (Kocks et al 1993). This pattern of ActA redistribution through bacteria division is similar to the pattern of actin filament redistribution described abov (Tilney & Portnoy 1989). An unexplained disparity between actin and Act localization is that ActA is polarized even on bacteria grown ouside of hos cells, whereas initially the actin clouds around bacteria in infected cells ar symmetric. When the ActA protein is expressed in mammalian cells, it local izes primarily to mitochondria (Pistor et al 1994). It is tempting to speculat that this preferential localization is because the mitochondria still retain som of the localization machinery derived from their bacterial endosymbiont an cestors. The actin cytoskeletons in ActA-transfected mammalian cells ar abnormal, with foci of polymerized actin around the mitochondria (Pistor e al 1994). Conclusive proof that ActA is the bacterial protein primarily respon sible for *L. monocytogenes* actin-based motility came with the recent demon strations that the protein expressed either in the nonpathogenic *L. innocu* (Kocks et al 1995) or in purified form (Smith et al 1995) is sufficient to produc comet tail formation and actin-based motility in cytoplasmic extracts. Asym

metric distribution of the protein is important for the efficient generation of comet tails and actin-dependent movement (Smith et al 1995).

The amino acid sequence of ActA shows no overall similarity to that of any other known protein. There are some short stretches that show a low degree of homology to known cytoskeleton-associated proteins including vinculin and caldesmon (Domann et al 1992, Kocks et al 1992; reviewed in Sheehan et al 1994), but it is not yet known whether these homologies are of any functional significance. Upon inspection of the sequence, the most striking structural feature of the ActA protein is a short repeated peptide rich in proline and acidic residues (Kocks et al 1992). Microinjection of a synthetic peptide corresponding to one proline-rich repeat of ActA into infected cells stops bacterial movement (Southwick & Purich 1994). This effect may be due to nonspecific effects of the peptide on the actin cytoskeleton, because microinjection of any other agent that disrupts normal actin dynamics also causes movement to cease (Sanger et al 1995). A systematic study of a series of internal deletion mutants has revealed that there are two separable domains of ActA required for interaction with the actin cytoskeleton: the first for filament nucleation, and the second for rapid actin filament dynamics and efficient tail formation. The second domain includes the entire proline-rich region of the protein and is also necessary for the association of profilin with the surface of intracellular bacteria (GA Smith, JA Theriot & DA Portnoy, in preparation). These results are consistent with the suggestion that profilin is involved in rapid filament growth at the bacterial surface (Theriot et al 1994), although there is still no evidence that profilin can bind directly to the proline-rich region of ActA. It is not yet known whether a host actin nucleating protein associates with the first domain of ActA, or whether this domain might by itself have some nucleating activity in the cytoplasmic environment. ActA does not appear to be sufficient to induce nucleation of pure actin.

There is no direct evidence that any *L. monocytogenes* genes other than *actA* contribute to the association with actin or comet tail formation. A single strain generated by transposon mutagenesis has been reported to retain the ability to nucleate actin filaments in the cytoplasm of infected cells without efficiently forming comet tails (Kuhn et al 1990). This observation suggests that a second bacterial product is required for tail formation, but this is difficult to reconcile with the fact that ActA alone is sufficient to generate cytoplasmic comet tails in heterologous systems (Smith et al 1995, Kocks et al 1995). The site of transposon insertion in this interesting mutant strain has not been reported.

The *icsA* gene (also called *virG*) in *S. flexneri* is the functional equivalent of *actA*. Mutant strains in which this gene has been disrupted are competent to invade host cells and multiply, but they form microcolonies in the cytoplasm and do not spread between cells (Makino et al 1986, Bernardini et al 1989). Despite their phenotypic similarity, there is no detectable sequence homology

between IcsA and ActA. IcsA is an outer membrane protein with a standard signal sequence; unlike the Ipa proteins it does not require the virulence-specific secretion apparatus to be properly expressed on the cell surface. Expression of IcsA is regulated at the transcriptional level by the bacterial growth phase, such that higher levels of IcsA are expressed when the bacteria are dividing rapidly (Goldberg et al 1994). Similarly, within infected cells, movement of the bacteria is cell-cycle dependent and most likely to be initiated while the bacteria are dividing (Prévost et al 1992, Goldberg et al 1994). *E. coli* expressing IcsA (and no other *S. flexneri*-specific proteins) are capable of generating actin-rich comet tails and moving in cytoplamic extracts (Goldberg & Theriot 1995, Kocks et al 1995). Therefore, IcsA is the primary *S. flexneri* protein responsible for actin-based motility. The mechanisms of interaction between IcsA and the host cell cytoskeleton have not been determined. Purified IcsA has been reported to show a low level of ATPase activity (Goldberg et al 1993), but it is not known how this is involved in its function. IcsA is a potential substrate for cAMP-dependent kinase in the host cell. Alteration of the putative phosphorylation site does not result in an inhibition of bacterial motility, indeed, the bacteria spread somewhat more efficiently (d'Hauteville & Sansonetti 1992).

Like ActA, IcsA is a member of the small class of bacterial surface proteins (reviewed in Maddock et al 1993) that are distributed in a polarized fashion (Figure 5) (Goldberg et al 1993). *S. flexneri* strains that are deficient in synthesis of the O-antigen of lipopolysaccharide are unable to properly polarize IcsA (Sandlin et al 1995) and are deficient in tail formation, actin-based motility, and cell-to-cell spread, although they can nucleate actin filament clouds (Rajakumar et al 1994, Sandlin et al 1995). When IcsA is expressed in *E. coli*, it is distributed uniformly over the entire surface, and these bacteria are substantially less likely to form comet tails than are intracytoplasmic *S. flexneri* (Goldberg & Theriot 1995). Thus polar expression of IcsA requires

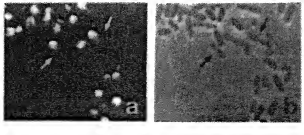


Figure 5 Asymmetric distribution of IcsA on the surface of *S. flexneri*: (*a*) indirect immunofluorescence, (*b*) phase-contrast. Arrows mark dividing bacteria. Micrograph courtesy of M Goldberg.

O-antigen and possibly other factors not expressed by *E. coli* and may be involved in the efficient formation of the asymmetric comet tail.

L. monocytogenes and *S. flexneri* seem to have independently evolved similar solutions to the problem of generating actin-based motility in host cell cytoplasm. Both express a single surface protein (ActA or IcsA) that interacts with the actin cytoskeleton and is more highly concentrated at one pole of the bacterium. The bacterial proteins need only to induce asymmetric nucleation and rapid elongation of actin filaments at the bacterial surface; the comet tail structure is then formed by actin cross-linking proteins in the host cell cytoplasm. Because IcsA and ActA show no sequence similarity, it is likely that the two proteins act by tapping into different steps along a common actin-regulatory pathway.

Generation of Force and Movement

It is still an open question how the actin filament rearrangements dictated by these bacterial surface proteins lead to force generation and motility. There are at least two plausible models. In the first, a molecular motor protein such as a myosin is bound to the bacterial surface. As actin filaments grow with their barbed ends pointing towards the bacterium, the barbed end–directed motor activity of the myosin pushes the bacterium away from the growing tail. This opens up space for further polymerization to occur. The rate of bacterial movement generated by the motor protein cannot outrun the rate of polymerization of the actin filament tracks. No evidence has been found for the association of myosin II with either the bacterial surface or the comet tail (Dabiri et al 1990), but it is possible that one or more of the many myosin I and myosin V isoforms present in mammalian cells (reviewed in Cheney et al 1993) may associate with the bacteria. There are as yet no other biological actin-dependent force-generating systems that have been conclusively proven to involve no myosins.

The second model is that actin filament polymerization itself provides the force for bacterial propulsion. Because actin is an ATPase, and ATP hydrolysis is associated with monomer addition onto a filament end, it is theoretically possible that polymerization may generate mechanochemical force (Hill & Kirschner 1982), but the mechanism of force generation is not obvious. An interesting explanation has been suggested by Peskin et al (1993). According to this "Brownian ratchet" hypothesis, the tail would act to rectify the random thermal movements of the bacterium, preventing backward diffusion but permitting forward diffusion. Occasionally, the excursion of the bacterium would be large enough to permit addition of another actin monomer onto the end of preexisting filaments; this would result in a new equilibrium position for the bacterium. The irreversibility of the system is guaranteed by the actin ATPase. Given reasonable assumptions about the concentration of monomer available

and the cytoplasmic viscosity, the rates of *L. monocytogenes* and *S. flexneri* movement observed are well within the possible range for this mechanism. One objection to this explanation is that the rate of bacterial movement does not appear to be directly related to the density of filaments in the tail (Nanavati et al 1994). It is not yet possible to distinguish between the two models. Resolution of this question may await full reconstitution of bacterial actin-based motility using only purified proteins.

CELL-TO-CELL SPREAD

In an infected host animal, the major function of bacterial actin-based motility appears to be an increase in efficiency of cell-to-cell spread. However, for the bacteria to actually reach the cytoplasm of a neighboring cell, several other events must follow the initiation of intracellular motility. First, the moving bacteria must form protrusions that allow them to move some distance from the bulk of the infected cell. Next, the protrusions must invade a neighboring cell. Finally, the bacteria must degrade the double membrane separating them from the cytoplasm of the second cell. Again, this sequence of events requires close cooperation between the pathogen and the infected host cell.

Formation of Protrusions

When locomoting bacteria reach the edge of the cell, they often push out membrane-bound protrusions without significantly slowing. This indicates that the deformation of the plasma membrane is not energetically costly. Although cell surfaces are usually fairly rigid, the rigidity is due to the underlying actin-rich cortex and not to the membrane itself. Thus a latex bead adhering to membrane proteins can be pulled away from the surface of a cell to create a thin cylindrical membrane protrusion, as long as the membrane proteins are not anchored to the actin cytoskeleton (Schmidt et al 1993). The protrusions formed by motile *S. flexneri* and *L. monocytogenes* can be very long, up to tens of micrometers in infected macrophages, although typically membrane protrusions pulled out with latex beads "snap back" within a few micrometers. This raises the possibility that the bacteria may need to specifically recruit extra membrane to the surface of the protrusions.

No mutant strains of either *L. monocytogenes* or *S. flexneri* have been described that are capable of making comet tails but not protrusions. Thus it is likely that the close interactions between the comet tail and the membrane in protrusions are mediated by host cell proteins rather than bacterial proteins. These interactions may involve the adhesion plaque proteins vinculin and talin, which are present along protrusions (Kadurugamuwa et al 1991, Dold et al 1994), and other yet unidentified actin- and membrane-binding proteins. *S. flexneri*-infected cells that do not express calcium-dependent cell adhesion

molecules cannot make structurally normal protrusions (Sansonetti et al 1994), which supports the hypothesis that components of the cell adhesion apparatus contribute to the architecture of the protrusions.

Endocytosis of Protrusions by Neighboring Cells

Entry of the bacteria-containing protrusions into nearby cells essentially amounts to one cell phagocytosing a bit of its neighbor. Because such an event would not occur under normal circumstances, we might expect that the bacteria are altering the extracellular surface of the protrusion in some way to induce the second cell to initiate phagocytosis. However, there is no evidence that this is the case. It is possible that the force generated by the bacterium moving at the tip of the protrusion is sufficient to push it into the second cell, in what might be thought of as an inverted protrusion event. In a tissue culture model of *S. flexneri* spread, calcium-dependent cell adhesion molecules must be present on both the sending and the receiving cell for efficient uptake of the protrusions, thus close adhesive contacts between the two cells do appear to be necessary (Sansonetti et al 1994).

Lysis of Double Membrane

Once the protrusions have been taken up by the neighboring cell, the bacteria are separated from the cytoplasm of the second host cell by two membranes: the plasma membrane of the first cell, with its cytoplasmic leaflet toward the bacteria, and the plasma membrane of the second cell. The membrane topography is different from that originally faced by the bacteria as they were internalized by the first cell; there, the host cell plasma membrane external leaflet was facing toward the bacteria. Because the complement of lipids and proteins exposed by the cytoplasmic leaflet is different from that exposed by the external leaflet, it is not surprising that the bacteria require different enzymes to disrupt the double endosome than they did to disrupt the original single endosome.

In *L. monocytogenes*, the broad-spectrum phospholipase encoded by *plcB* is important for the lysis of the double bilayer. Mutant strains in which *plcB* has been disrupted tend to accumulate in a double-membrane compartment (Vasquez-Boland et al 1992). The phospholipase is secreted in an inactive form and must be activated by a specific secreted metalloprotease, encoded by *mpl* (Domann et al 1991, Mengaud et al 1991c). It is likely that listeriolysin O also contributes to the secondary escape. Once the bacteria have entered the cytoplasm of the second cell, they are again able to associate with actin filaments, and the cycle of intra- and intercellular spread continues.

In *S. flexneri*, two genes specifically involved in cell-to-cell spread have been identified: *icsB* on the large virulence plasmid (Allaoui et al 1992a) and *vacJ* on the bacterial chromosome (Suzuki et al 1994). Mutants deficient in

the product of either gene tend to accumulate in the double-membrane compartment. The enzymatic activities of these two proteins are not known; neither shows any significant homology to the broad-spectrum phospholipase C of *L. monocytogenes*.

Induction of Apoptosis

When infectious bacteria are taken up by a host cell, they detect the change in their environment and respond by altering the transcriptional levels of multiple virulence genes. This communication works in both directions; the presence of the bacteria also alters transcription of some genes in the infected host cell. *S. flexneri* is capable of initiating events in the host cell that lead to apoptosis, with the typical morphological changes and DNA degradation seen in other examples of programmed cell death (Zychlinsky et al 1992). In the intestine of an infected animal, this results in rapid death of the phagocytes and other cells, which contributes to the abcess formation characteristic of *Shigella* dysentery. *ipaB* is required for causing host cell apoptosis as well as for internalization and escape from the membrane-bound compartment, but *ipaC* and *ipaD* are not apparently involved (Zychlinsky et al 1994). It is not known how IpaB (and possibly other *S. flexneri* proteins) initiate the apoptotic process. There is no evidence that *L. monocytogenes* is capable of inducing host cell apoptosis.

CONCLUSIONS: WHAT CAN *L. MONOCYTOGENES* AND *S. FLEXNERI* TEACH US ABOUT THE CELL BIOLOGY OF THE CYTOSKELETON?

L. monocytogenes and *S. flexneri* are only two examples of widely studied bacterial pathogens, but they can offer insights into long-standing problems in several areas of cyoskeletal cell biology. At their initial contact with host cells, they signal through the host plasma membrane to cause actin rearrangements resulting in phagocytosis, even if the cells are not normally phagocytic or do not normally produce protrusive actin structures. This phenomenon may be a useful model system for other types of transmembrane signaling that result in cytoskeletal rearrangements, including chemotaxis and pathfinding by neurite growth cones. Different types of bacterial pathogens are able to use transmembrane signaling to elicit morphologically and dynamically distinct actin structures in the same host cells; for example, *L. monocytogenes* induce phagocytosis, *Salmonella* spp. cause dramatic ruffling, and enteropathogenic *E. coli* induce pedestal formation, all on intestinal epithelial cells. By understanding the signaling used by the bacteria we may learn about the ways mammalian cells can respond appropriately to different external cues, even though they all elicit actin polymerization near the membrane.

The actin-based movement of intracytoplasmic *L. monocytogenes* and *S. flexneri* has already become a widely used model system for whole cell motility. The biochemical investigation of lamellipodial and filopodial protrusion has been hampered by the obligatory role of the cell plasma membrane in generating the structures and producing force. The movement of the bacteria, however, has been reconstituted in cell-free cytoplasmic extracts and should be more amenable to traditional biochemical techniques. Additionally, now that a single bacterial protein from each species has been identified as being sufficient to generate actin-based motility, the bacterial proteins can be used as molecular handles to identify the rest of the motile machinery.

Formation of protrusions by the bacteria may be a useful model for interactions between the actin cytoskeleton and the plasma membrane. Several examples of actin-membrane contacts have been very well studied, including adhesion plaques and microvilli. However, relatively little is known in mammalian non-erythrocyte cells about how actin filament structures generally attach to the cell membrane. The rapidly forming bacterial protrusions may provide some novel information.

These are only two of the better-understood examples of bacterial pathogens that have drastic effects on host cell actin. Other infectious bacteria have developed different strategies for interacting with host cells. The study of host-pathogen interactions will continue to yield new approaches to solve old and intractable problems in cell biology, as more human cell biologists begin to listen to the ancient knowledge of the infectious bacterium.

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